

Compositions and Their Uses

5 The present invention relates to the field of transplantation and, in particular, to preventing hyperacute rejection of xenografts or at least reducing the extent or rate of rejection.

10 Transplantation of a pig organ (e.g. heart, kidney) into a primate triggers a rapid (sub-10 minute) hyperacute rejection in the recipient which destroys the donor tissue.

15 The first step in hyperacute rejection is considered to be the binding of pre-formed "xenogenic natural antibodies" (XNA) of the recipient to the blood vessels of the donor tissue which in turn activates the complement system of the recipient. There is a strong evidence that the major target of these antibodies is galactose  $\alpha 1,3$ galactose (Gal $\alpha 1,3$ Gal) (Sandrin et al, *Proc Natl Acad Sci USA* 90: 11391 (1993)). This is a carbohydrate which is widely expressed on pig tissue, but absent from humans (Galili et al, *Proc Natl Acad Sci USA* 84: 1369-1373 (1987)). This disaccharide is a terminal modification of the oligosaccharide chains displayed by  
20 cell-surface glycoproteins and glycolipids. A number of porcine endothelial proteins which bind XNA, and therefore probably carry Gal $\alpha 1,3$ Gal, have been identified (Platt et al, *Transplantation* 57: 327-335 (1994)).  
25

30 The majority of XNAs directed against porcine tissues are known to bind specifically Gal $\alpha 1,3$ Gal. Oligosaccharides containing this structure (B-trisaccharide: Gal $\alpha 1,3$ Gal $\beta 1,4$ GlcNAc; B-disaccharide: Gal $\alpha 1,3$ Gal) are the best inhibitors of the cytotoxic effects of human serum

09692249-101900

on porcine endothelial cells (Neethling et al, Transplantation 57: 959-963 (1994)). Other  $\alpha$ -galactosylated sugars (not 1,3-linked) do inhibit, but far less efficiently.

5

Cooper and co-workers have examined how infusion of the  $\alpha$ -galactosylated sugars arabinogalactan and melibiose into baboons affects the ability of serum from these animals to kill pig endothelial cells in vitro (Ye et al, Transplantation 58: 330-337 (1994)). A level of protection was afforded, but not sufficient to significantly extend the life of a heterotropic pig heart xenotransplant. In addition, high levels of carbohydrate infusion can make the animals ill (diuresis, respiratory distress).

15

The group of Cooper et al have also attempted to inhibit antibody-mediated lysis of porcine cells in vitro with B-disaccharide-polyacrylamide (PAA) conjugates commercially available from Syntesome GmbH, Fine Biochemicals, Heimdall Str. 4, D-81739 Munich, Germany. (Riebin et al, Xenotransplantation 2: 98-106 (1995)). The disaccharide can be attached to polyacrylamide at a range of substitution densities, which vary in their ability to protect pig tissue from attack. However, no direct comparisons of the protective capability of the PAA conjugate and free disaccharide have been published.

20

25

WO 93/03735 discloses various methods and compositions which are said to be useful for attenuating antibody-mediated xenograft rejections. In one embodiment, xenoantigens are coupled to solid supports and are indicated as being useful for perfusion of a patient's blood in order to remove xenoantibodies. Examples of

30

00692249 "101900

suitable solid supports are said to be silica, synthetic silicates such as porous glass, biogenic silicates such as diatomaceous earth, silicate containing minerals such as kaolinite, and synthetic polymers such as polystyrene, polypropylene and polysaccharides.

According to the present invention there is provided a synthetic conjugate of a protein and a plurality of epitopes, for use in medicine; wherein said epitopes are capable of being bound by xenogenic natural antibodies.

The term "protein" is used herein to include moieties with peptide bonds, such as peptides (e.g. polylysine), polypeptides and complete proteins. It should therefore not be construed in an unduly limiting manner. If desired, the characteristics (e.g. the charge characteristics) of a protein for use in the present invention may be modified. For example, methoxyethoxyacetylation of the epsilon-amino group of lysine in poly (L-lysine) reduces non-specific interactions between it and tissues as a result of the polypeptide's cationic charges (Gasho, A., et al, *Biol. Pharm. Bull.* 17:275-282 (1994)).

The term "synthetic" is used here to indicate that the conjugate does not occur naturally.

The term "conjugate" indicates that the protein and epitopes are linked together.

The term "xenogenic natural antibody" is used here to include both:

- (a) antibodies which are already present in a host

006707" 64226960

and which can participate in rejection of xenografts in that host. (These antibodies are thought to be involved in hyperacute rejection of xenografts.)

5 and (b) antibodies which are newly synthesised in a host in response to the present of a xenograft in that host. (These are sometimes referred to as "xenoreactive elicited antibodies" and are thought to be involved in acute rejection of xenografts).

10

The present inventors have discovered that a conjugate according to the present invention is highly effective in binding to xenogenic natural antibodies and is particularly effective in binding IgM. This finding is significant since IgM is implicated in the early stages of rejection and is generally believed to be the most important immunoglobulin class in this process. Blocking of IgM binding to epitopes expressed on a xenograft is therefore a significant advance in preventing or alleviating rejection.

15

20

Since a large number of epitopes can be bound to a single protein molecule the total number of molecules required to present a given number of epitopes to a patient's immune system can be significantly reduced relative to the use of individual epitopes. The conjugate can be used to avoid osmotic disturbances or to result in fewer osmotic disturbances in a patient (in comparison to the free sugar) since osmolarity is directly related to the concentration of molecules in solution.

25

30

Preferably the epitopes will bind to human xenogenic natural antibodies involved in the rejection (e.g. acute or hyperacute rejection) of xenografts taken from pigs.

006T0T" 54226960

However, the epitopes may bind to human xenogenic natural antibodies involved in the rejection of xenografts taken from other animals. Preferred epitopes are therefore not normally present in the human adult. Particular epitopes which can be used are discussed later on.

The present invention encompasses a method of preventing rejection of a xenograft, or at least of reducing the extent or rate of rejection, by administering the conjugate to a recipient of the xenograft. (This may be done before, during and/or after implanting the xenograft. Preferably it is done before implantation, e.g. up to twenty-four hours before a transplant operation. Booster doses may then be administered subsequently). The conjugate can bind to xenogenic natural antibodies present in the recipient and can therefore block the binding of these antibodies to epitopes present on the xenograft. This blocking effect removes many free xenogenic natural antibodies from the circulation of a recipient and these antibodies are therefore not free to activate complement by binding to epitopes present on the xenograft. Complement activation is believed to be a major factor in hyperacute rejection of xenografts and the blocking effect which can be achieved using the conjugate of the present invention is therefore of major significance. Indeed even if this blocking effect is only short-lived (e.g. if it lasts only for a few days) then there are indications in the art that it can still be effective since accommodation of a xenograft may occur. This is discussed by Rieben et al (*supra*), where an analogy is drawn with the ABO system. In any event it is possible to monitor a recipient and to provide additional conjugate of the present invention when necessary.

00692249 "101900

Conjugates of the present invention can function as tolerogens i.e. as molecules which can reduce immunogenicity in response to subsequent challenge with a hapten. Thus administration of conjugates of the present invention prior to inserting the pig xenograft may inhibit an antibody response to the graft itself, through tolerance. When a graft is already in place, the conjugate may be administered to prevent an antibody response to the graft by inhibition (i.e. by reducing the production of anti-hapten antibody).

(By way of background, the work of Dintzis (e.g. Dintzis, R.Z., et al, *J. Immunol.* 131:2196-2203 (1983)) with multivalent polyacrylamide conjugates can be referred to. This has demonstrated that conjugates presenting multiple haptenic groups can be either immunogenic (that is, will stimulate anti-hapten antibody production) or inhibitory (reduce the production of anti-hapten antibody in the presence of immunogenic conjugates). Whether the conjugate is immunogenic or inhibitory depends on the conjugate size and hapten density. If administration of an inhibitory conjugate before challenge with an immunogenic conjugate reduces the immunogenicity of the latter, the inhibitory molecule is termed tolerogenic. All these effects (immunogenicity, inhibition, and tolerogenicity) are thought to operate through competition at the hapten receptors on B-cells. Tolerogenic inhibitory molecules are envisaged as persisting at the B-cell receptor in spite of removal from the surrounding environment.)

The conjugates used in the present invention can be administered to a patient by any appropriate technique, but is preferably administered by infusion (by means of

006707-64226960

5 a drip). Dosage levels can be determined by those skilled in the art and will depend upon factors such as the mass of the patient, the size of the xenograft, the size of the protein present in the conjugate, the number of epitopes present in the conjugate, the spacing of such epitopes, etc. Without being bound by theory, a typical dosage for the B-trisaccharide-HSA referred to in the Example might be 0.1mg/ml to 5mg/ml final concentration in the serum. This range is derived from the concentration of conjugate observed to fully inhibit the lysis of endothelial cells *in vitro* by 10 $\mu$ g/ml XNA IgM (see Figure 3, which will be described later), which is approximately 0.5mg/ml. 10 $\mu$ g/ml represents the approximate concentration of XNA IgM expected in the serum. Dosage ranges for other conjugates of the present invention can be approximated using a similar assay procedure. Animal experiments may then be used to determine dosage levels more accurately.

20 As indicated above, a patient can be monitored and additional doses of the conjugate can be administered when necessary. Monitoring can be performed by the following technique: serum from a patient is tested *in vitro* for levels of soluble XNA antibodies (primarily of the G and M classes). The serum is first fractionated into individual immunoglobulin classes. These are then administered to separate microtitre plate wells coated with Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc-HSA. Bound antibody is detected with reporter enzyme conjugated anti-human IgG or -IgM antibody.

The present invention can also be used to provide an immunoadsorbent which is located outside of a subject's body and which acts to remove xenogenic natural

006707 6426950

antibodies of the subject which are involved in rejection of xenografts. Desirably the immunoadsorbent will be immobilised - e.g. it will be present in a column. Thus, xenogenic natural antibodies can be removed from blood or at least their titre can be reduced. This is advantageous since a transplant patient's own blood can be treated using this technique and then returned to the patient. Another benefit of this technique is in providing stocks of blood from blood donors, which can be packaged for later use and used in the treatment of transplant patients. Such treated blood is within the scope of the present invention.

The present invention also provides an apparatus in which at least one conjugate of the present invention is located in a chamber provided with an inlet and an outlet. Blood can be treated by passing it through this chamber via the inlet and outlet.

Preferably the protein component of the conjugate is a human protein. The term "human protein" is used here to include proteins which occur naturally in humans, as well as such proteins or derivatives thereof having the same or a substantially similar biological activity. Such proteins may be produced naturally or may be produced synthetically (e.g. by chemical synthesis or by using recombinant DNA technology).

Desirably the protein is a component of blood. For example, it may be serum albumin (which may be in natural or recombinant form) or another generally inert native or recombinant protein, which is non-antigenic and non-immunogenic in the un-conjugated state and which is not a ligand for a human receptor molecule.

006707-101900



Preferably the protein is soluble (rather than membrane bound).

5 The protein may be associated with a plurality of epitopes to provide a conjugate for use in the present invention by any appropriate technique. Preferably the epitopes are covalently bound to the protein. This may be done using a spacer molecule.

10 A reaction which can be used to introduce a three atom spacer is a Michael addition between an acroylylate derivative of a sugar and a protein, e.g. HSA (Roy et al, *J Chem Soc Chem Commun* 1709-1711 (1990)). The reaction consists of three main steps:

- 15
- i. Amination of a reducing sugar (e.g. B-trisaccharide):- incubation in saturated ammonium hydrogen carbonate for 3 days at 37°C.
  - 20 ii. Acryloylation:- use acryloyl chloride in the presence of  $\text{Na}_2\text{CO}_3$ , in a methanol-water solvent.
  - iii. After extensive lyophilisation, and purification of the derivatised sugar by gel filtration and reversed phase-HPLC it is coupled to HSA in 0.1M  $\text{Na}_2\text{CO}_3$ , pH 10.0 for 2 days at 37°C. The
  - 25 product of the reaction is sugar-NH-CO-CH<sub>2</sub>-CH<sub>2</sub>-NH-HSA.

(The same basic reaction can be used to couple a sugar to polyacrylamide.)

30 Alternatively a sugar with a two carbon allyl linker in place can be coupled to a protein by a method using reductive ozonolysis followed by reductive amination with sodium cyanoborohydride (Bernstein, M.A. and

005707-101900

Laurance, D.H., *Carbohydr. Res.* 78 (1980)).

5 The epitopes will desirably include as a minimum structure the smallest ligand necessary for binding of XNAs, which is galactose linked in an alpha-configuration. Thus the epitopes are preferably saccharides and desirably are oligosaccharides, or mimics of these saccharide structures.

10 The epitopes are desirably saccharides and are preferably oligosaccharides. Mimics of any such saccharides or oligosaccharides are included within the scope of the present invention. The term "mimic" refers to any  
15 structure which acts in substantially the same manner as a saccharide or oligosaccharide in binding to an antibody.

Such mimics are known in the art (and are sometimes referred to as mimetics). For example, Shikhman A.R. and  
20 Cunningham M.W., (*J. Immunol.* 152(9):4375 (1994)) describe the use of cytokeratin peptides which act as mimics of sugar epitopes by blocking anti-GlcNAc antibodies; Vaughan et al (*Xenotransplantation* 3:18-23 (1996)) describe a synthetic octapeptide (DAHWESWL) which  
25 mimics the epitope Gal $\alpha$ (1,3)Gal; and Koogman et al describe a different peptide (SSLRGF) which also mimics the epitope Gal $\alpha$ 1,3Gal.

30 The epitopes may include sulphate groups, sialic acid and  $\alpha$ -galactose (or mimics thereof).

The conjugate may be a neoglycoprotein (a non-glycosylated protein to which saccharides of a defined structure have been attached). Neoglycoproteins are

006707 6422650

discussed by Adler et al (*J Biol Chem*, 270: 5164-5171 (1995)) and have been used for probing carbohydrate-protein interactions. They can be produced by enzyme catalysed reactions (e.g. using glycosyltransferases or glycosidases) or by chemical synthesis techniques. The methodology for chemical glycosylation of human serum albumin (HSA) and bovine serum albumin (BSA) is well established, as is the chemical synthesis of B-trisaccharide (Jacquinet et al, *JCS Perkin T*: 326-330 (1981)). Indeed a series of suitable Gal $\alpha$ 1,3Gal based neoglycoproteins are available from Dextra Laboratories Ltd, Reading, UK. This company produces HSA-conjugated Gal $\alpha$ 1,3Gal and HSA conjugated-Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc. Both of these can be obtained as either the 3- or 14- atom spacer molecules. Similarly the company produces four BSA-based neo-glycoproteins (HSA and BSA refer to human serum albumin and bovine serum albumin respectively).

Where it is desired to use xenotransplants from pigs, preferred epitopes for use in producing the conjugate include two galactose moieties. Desirably the two galactose moieties are linked by an  $\alpha$ 1,3 linkage. Thus Gal $\alpha$ 1,3Gal is a favoured epitope for use in the present invention. This epitope may be present as part of a larger oligosaccharide structure. For example, it may be part of a trisaccharide: e.g. Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc. Other structures which can be used include:-

- 1) Multi-antennary oligosaccharides which may present up to 4 Gal $\alpha$ 1,3Gal termini. These oligosaccharides can be readily purified from natural sources e.g. bovine or porcine thyroglobulin.
- 2) Glycolipid derived  $\alpha$ -galactosylated oligosaccharides.

006707 64226960

3) Synthetic glycosphingolipids bearing the correct terminal structure. Thus creating a neo-sphingoglycoprotein.

5 The disaccharide Gal $\alpha$ 1,3Gal may itself be used.

As alternatives to the epitopes discussed above, other epitopes could be used. These include: oligosaccharides with terminal galactose  $\alpha$ -linked to a subterminal residue which need not be galactose. The  $\alpha$ -linkage need not be a 1,3 linkage. Examples are: melibiose: Gal $\alpha$ 1,6Glc; raffinose : Gal $\alpha$ 1,6(Fuc  $\alpha$ 1,4) Glc and arabinogalactan.

Where xenografts are used from animals other than pigs then appropriate epitopes in respect of those animals can be used to prepare a conjugate of the present invention. In any event, Gal $\alpha$ 1,3Gal is expressed widely among non-primate mammals and New World Monkeys and is likely to be a major epitope in xenotransplantation of organs from a wide variety of mammals. However, as in the case of the pig, other epitopes may be used. Furthermore other species may present unique epitopes which will react with human XNAs. As with Gal $\alpha$ 1,3Gal none of these epitopes are likely to be expressed by the adult human.

Various preferred epitopes which can be used in the present invention are the carbohydrates given in the list below. (However other epitopes can of course be used (e.g. the xenoantigens described in WO93/03735).)

30

#### List of preferred epitopic structures

(i) Any carbohydrate terminating in Gal $\alpha$ 1,3Gal (as previously claimed) including blood group B-related

00652249-101900

oligosaccharides, the pentasaccharide  
 $\text{Gal}\alpha 1,3\text{Gal}\beta 1,4\text{GlcNAc}\beta 1,3\text{Gal}\beta 1,4\text{Glc}$ ,  
 $\text{Gal}\alpha 1,3\text{Gal}\beta 1,3\text{GalNAc}\beta 1,4\text{Gal}\beta 1,4\text{Glc}$  and blood group I-  
 5 active carbohydrates (Dabrowski, U., et al, *J. Biol.*  
*Chem.* 259:7648-7651 (1984)).

(ii) n-glycolylneuraminic acid. This is a  
 monosaccharide expressed in pig tissue but not on normal  
 human tissue; however it has been described on human  
 10 tumours (Fukui, Y., et al, *Biochem. Biophys. Res. Commun.*  
 160:1149-1154 (1989)). In these respects n-  
 glycolylneuraminic acid is similar to  $\text{Gal}\alpha 1,3\text{Gal}$ .

(iii)  $\text{Gal}\alpha 1,\text{XGal}$  where X could be 2, 4, or 6. There  
 15 is some evidence that some XNAs recognise these  
 structures (Wieslander, J., et al, *Glycoconjugate J.*  
 7:85-100 (1990)).

(iv) Blood group A or A-like substances. There is  
 20 evidence that these structures are expressed on pig heart  
 (Cooper, D., et al, *Transpl. Immunol.* 1:198 (1993)) and  
 kidney (Jalali-Araghi, K., and Macher, B.A.,  
*Glycoconjugate J.* 11:266-271 (1994)). Experimental  
 25 evidence is presented in Table 1 herein that anti-A  
 antibodies are elicited in primate recipients of kidney  
 xenografts:

Infusion of A and B carbohydrates has been performed in  
 the past to control pathologies resulting from blood  
 30 group incompatibility (Romano, E.L., et al,  
*Transplantation Proceedings* 19:4475-4478 (1987)).

(v) T- and T-related antigens. Humans have  
 significant titres of antibody against the T-antigen.

0069249-101900

(Thomsen Freidenreich antigen = Gal $\beta$ 1,3GalNAc-). Although this does not normally lead to problems as the disaccharide - at least in pig and human lymphocyte membranes - is cryptic, that is normally capped by non-immunogenic neuraminic acid (Newman et al, *Eur. J. Biochem.* 64:373-380 (1976)). It is possible that this capping is not complete in all tissues carried in the porcine xenograft. Furthermore, genetic manipulation of xenografts e.g. the ablation of terminal Gal $\alpha$ 1,3Gal may expose T-antigen.

Humans also carry natural antibodies against the Tn antigen (GalNAc-O-Ser/Thr) a normally cryptic antigen which can be generated by failure of the tissue to add an overlying galactose (Thurnher et al, *J. Clin. Invest.* 91:2103-2110 (1993)).

The T-antigen is likely to be a common substituent of mucin (O-linked) oligosaccharides. To test for anti-T antibodies in the serum of primate recipients of pig cardiac xenografts.

IgG and IgM subclasses were incubated with fixed endothelial cells in the presence of high molecular weight pig stomach mucin treated to remove  $\alpha$ -galactosyl groups and neuraminic acid (Table 2). The removal of  $\alpha$ -galactose by coffee bean  $\alpha$ -galactosidase was confirmed by IB-4 lectin binding studies (date not shown). In a preliminary experiment we found that the residual binding activity, probably due to T-antigen recognition, consistently accounted for ~15-20% of the antibody binding to fixed endothelial cells and was confined to the IgM class (unlike anti-Gal $\alpha$ 1,3Gal, which can be of either IgG or IgM class).

005707" 54225950

(vi) poly-N-acetyllactosamine and lactosamine (-Gal $\beta$ 1,XGlcNAc-)n where n=1 or >1 and X may be 3 or 4. Although these structures are common modifications of human oligosaccharides, modified versions with A- B- and B-like termini may be particular to pigs. Not only will these oligosaccharides bind natural antibodies, they may be a target for lactosamine binding lectins (LBLs, (Feizi et al, *Biochemistry* 33:6342-6349 (1994)) which could attract inflammatory cells to the xenograft.

(vii) 3-sulphated galactose (SO<sub>4</sub>-3Gal $\beta$ -). This has been previously described as a potential xenoantigen (Samuelsson and Cairns, in *Complex Carbohydrates in Drug Research*, Alfred Benzon Symposium 36, 368-379. ed. Bock, Clausen (1994)) since it is expressed in normal human tissues as a cryptic antigen.

(viii) peptide epitopes which compete with Gal $\alpha$ 1,3Gal for binding to xenogeneic natural antibodies. These may be peptide sequences determined by phage display techniques or naturally occurring sequences. An example is a sequence or sequences on cat skeletal muscle superfast myosin which can bind a monoclonal anti-gal $\alpha$ 1,3Gal monoclonal antibody (Kirkeby, S., *Cell Tissue Res.* 283:85-92 (1996)).

(ix) N-acetyl- $\beta$ -D-glucosamine (GlcNAc). Cooper has identified anti-GlcNAc specificity among human antibodies eluted from pig hearts (Cooper, D., et al, *Transpl. Immunol.* 1:198 (1993)). however it is well known that human serum contains a natural antibody against GlcNAc (Emmrich et al, *J. Exp. Med* 161:547 (1985)) which may be cross-reactive with keratin (Shikhman and Cunningham, 1994). In the latter paper it was commented that this

anti-carbohydrate antibody only bound BSA-conjugates carrying a minimum of 20 GlcNAc residues. Albumin has a distinct advantage over many other potential "scaffolds" in that it is big enough to carry a large number of epitopes, and will still be soluble.

In a preferred embodiment of the present invention the conjugate comprises an epitope capable of being bound by a xenogenic natural antibody as well as a moiety which binds to liver hepatocytes (desirably with high specificity). This moiety preferably comprises a terminal  $\beta$ -linked galactose since this binds to a hepatic protein/asialoglycoprotein receptor found on hepatocytes (see Virgolini et al, *Nucl. Med. Commun.* 12:507-517 (1991)). The conjugate, together with xenogenic natural antibodies to which it is bound, can be cleared from circulation via the liver in a rapid manner.

The conjugate of the present invention may be provided in a kit, optionally including instructions for its use in preventing or alleviating rejection.

It will normally be provided in sterile form as a pharmaceutically acceptable composition, which may include additional ingredients, e.g. buffers, excipients, etc.

The conjugate may be provided in sterile saline or saline - dextrose, for example.

For ease of administration, it may be provided in unit dosage form. It may be in a form adapted for injection or infusion into a patient.

0065249-101900



5 The conjugate can be used for the manufacture of a medicament for use in preventing the rejection of a xenograft (e.g. a discordant xenograft) or at least in reducing the rate or extent of rejection. Desirably the medicament is used for preventing rejection of a xenograft from a human recipient.

10 It should be noted that although the conjugates of the present invention has been discussed above mainly in relation to xenotransplantation, they have other potential applications. For example, they may be useful in treating diseases in which xenoantigens (e.g. Gal $\alpha$ 1,3Gal) are implicated. The term "treating" is used herein to encompass prophylactic treatment as well as  
15 treatment of patients already having a particular disease.

20 Several human diseases involve recognition of Gal $\alpha$ 1,3Gal by anti-Gal $\alpha$ 1,3Gal antibody e.g. Chagas disease and Leishmania (Avila et al., *J. Immunol.* 142:2828-2834 (1989)) and ideopathic myelofibrosis (Leoni et al., *British J. Haematology* 85:313-319 (1993)). Towbin et al., *J. Exp. Med.* 166:419-432 (1987) report that a  
25 natural protein-linked conjugate Gal $\alpha$ 1,3Gal - mouse laminin is a very good inhibitor of the anti-Gal $\alpha$ 1,3Gal antibody elicited in Leishmaniasis sera and also normal human sera. Thus the present invention is of potential utility in treating Chagas disease, Leishmania and ideopathic myelofibrosis.

30 The present invention will now be described by way of example only, with reference to the accompanying tables and drawings; wherein:

00692249-101900

TABLE 1 shows the agglutination of human A+ and B+ erythrocytes (0.3%) by whole IgM fractions of pre- and post Tx sera.

5 Serum was collected from primate recipients of either pig cardiac xenografts (W544, W141, and W135) or pig kidney xenografts (T381 and V337). This was size fractionated to generate pools containing predominantly IgG or IgM. The IgM fractions were pre-adsorbed with human O erythrocytes to eliminate reactivity towards epitopes other than A- or B-substance. The IgM was then mixed with human A1 or B erythrocytes to a final dilution of 1:40 with respect to the original serum. Figures in parentheses indicate the number of days elapsed between surgery and collection of serum. "Pre-" denotes that the serum was collected before the xenografting operation. After 2 hours at room temperature the erythrocytes were inspected for signs of agglutination. Values in the 2nd and 3rd columns represent the lowest dilution of IgM (with respect of neat serum) that is able to cause complete agglutination of the erythrocytes. As controls, human A1 and B erythrocytes were also challenged with monoclonal anti-A or B group antibodies.

TABLE 2 shows the inhibition of antibody binding to fixed porcine aortic endothelial cells with agalactosyl-, asialo-high molecular weight fraction of pig stomach mucin.

IgG and IgM fractions from serum samples were mixed with either HSA or desialylated,

09692249-101900

5  $\alpha$ -galactosidase-treated pig stomach mucin (high  
 mol. weight fraction) so that the final  
 dilution of antibody with respect to serum was  
 1:12 and the final concentration of inhibitor  
 was 100  $\mu$ g/ml. 'W544' and 'W141' are two  
 cynomolgus monkey recipients of hDAF-expressing  
 pig heart xenografts. Numbers represent days  
 post transplant. 'Pre' denotes a pre-  
 10 transplant sample. After 2 hours at 4°C the  
 mixture were applied to wells coated with fixed  
 porcine aortic endothelial cells. After a 2  
 hour incubation period at room temperature,  
 bound antibody was detected with peroxidase-  
 15 conjugated anti-human IgM or IgG. Table 2  
 indicates the percentage change in binding of  
 immunoglobulin in the presence of the mucin as  
 opposed to HSA.

20 Inhibition of binding of XNA IgM to B-trisaccharide-HSA  
 by soluble sugars and glycoconjugates.

FIGURE 1 shows the results of a competitive binding  
 inhibition assay with conjugates which can be  
 used in accordance with the present invention,  
 as well as the results of this assay using free  
 oligosaccharides.

This figure indicates that, mole for mole,  
 B-trisaccharide-HSA is approximately 2 orders  
 of magnitude better at binding XNA IgM than  
 free B-trisaccharide even when the total number  
 of epitopes presented is taken into account.  
 One hypothesis is that the arrangement of  
 epitopes on the protein enables several arms of  
 the immunoglobulin to be bound at once,  
 increasing the affinity of the immunoglobulin.

00692249 "101900

Furthermore the protein scaffold may inhibit the free motion of the carbohydrate epitopes, increasing their antigenicity. These properties are not automatically shared by all conjugates (such as those mentioned in WO93/03735) and are all significant advantages of using a protein backbone.

A two stage chromium release assay on normal porcine endothelial cells (PAE 68#3) involving an initial incubation with serial dilutions of affinity isolated IgM or IgG followed by 1/8 baby rabbit complement.

FIGURE 2 shows the results of a two stage chromium release assay on normal porcine aortic endothelial cells (designated here as PAE-68#3) involving an initial incubation with serial dilutions of affinity-isolated IgM or IgG followed by 1/8 baby rabbit complement.

The amount of chromium released reflects the extent of complement-mediated lysis initiated by XNA binding.

A two stage chromium release assay on normal porcine endothelial cells (PAE-68#4) involving an initial incubation with either human affinity-isolated IgM (10 $\mu$ /ml) in the presence of four different sugars followed by 1/8 baby rabbit complement.

FIGURE 3 shows the results of a two stage chromium release assay on normal porcine endothelial cells (PAE-68#4) involving an initial incubation with either human affinity-isolated IgM (10 $\mu$ g/ml) or IgG (80 $\mu$ g/ml) in the presence of three different sugars or HSA-B-trisaccharide glycoconjugate followed by 1/8 baby rabbit complement

0969249-101900

Inhibition of binding of IgG or IgM from pooled human serum to fixed PAEC by pre-incubation with B-trisaccharide-HSA (HSA-3)

FIGURE 4 shows the results of a competitive binding inhibition assay in which total IgM and IgG fractions of human serum were mixed with varying concentrations of B-trisaccharide-HSA before incubating with fixed monolayers of cultured porcine aortic endothelial cells in wells of a microtitre plate.

In a second step, 1/200 peroxidase conjugated anti-human IgM and IgG antibody (Sigma) is added. Bound peroxidase is measured (vertical axis) and is related in a simple fashion to the amount of serum antibody attached to the porcine cells. The drop in immunoglobulin binding in the presence of the conjugate signifies the importance of Gal $\alpha$ 1,3Gal in the binding of pre-transplant IgM and IgG to porcine tissue.

Note that most of the binding of both IgG and IgM is through Gal $\alpha$ 1,3Gal recognition but the efficacy of the conjugate is superior against IgG. It may be the case that only partial inhibition of binding is sufficient to substantially reduce the cytotoxic effects of the antibodies.

w544: post Tx AAG IgG

FIGURE 5 shows the changes in anti- $\alpha$ Gal antibodies in the serum of a primate (w544, which is a cynomolgus monkey which had received a

0069249-101900

transgenic (human DAF expressing) pig heart xenograft and which was also on a low cyclophosphamide (immunosuppressant) regime) following grafting of a pig heart (genetically modified to be overcome hyperacute rejection).

IgM and IgG were initially separated by gel filtration chromatography and the anti- $\alpha$ Gal component in the IgG pool was measured by an ELISA method using B-trisaccharide-HSA as a target. The histogram shows an increase in anti- $\alpha$ Gal IgG concentration in the recipient's serum shortly before rejection of the xenograft. Also shown is the titre of generalised anti-pig antibody as measured by a complement-dependent pig red blood cell lysis assay.

Inhibition of binding of post-xenotransplant IgG to PAEC in the presence of B-trisaccharide-HSA

FIGURE 6 indicates how important recognition of Gal $\alpha$ 1,3Gal is in the binding of recipient post-xenotransplant immunoglobulin to porcine cells.

Post-xenograft serum from the recipient described in Figure 5 was applied to fixed aortic endothelial cell lines in the presence of either HSA or B-trisaccharide-HSA (0.2mg/ml). B-trisaccharide-HSA intervention had its greatest effect against pre-xenotransplant IgG and IgG from serum collected just before rejection. Thus, despite the continual presence of anti- $\alpha$ Gal antibody as measured by ELISA, the competitive strength of the conjugate in the presence of pig cells was

0069249 101900

variable, and probably related to affinity changes in the elicited antibody. It is encouraging that the anti- $\alpha$ Gal IgG peak that precedes rejection in this instance is relatively sensitive to competition with the conjugate.

Relative staining of PAEC by affinity-purified IgM

FIGURE 7 shows a comparison of the inhibitory potency of B-trisaccharide-HSA, other naturally  $\alpha$ -galactosylated glycoproteins and XNA IgM target xenogantigens.

Affinity purified anti-Gal $\alpha$ 1,3Gal IgM from human serum was applied to fixed porcine aortic endothelial cells (PAEC) after pre-incubation with either HSA, B-trisaccharide-HSA, murine EHS laminin, bovine thyroglobulin, or porcine platelet xenoantigens. The horizontal axis represents the amount of XNA IgM bound to the PAEC which is detected with horseradish peroxidase conjugated human anti-IgM antibody.

006707 " 64225950

Example

5 XNA were purified from 500 ml human serum by affinity chromatography on thyroglobulin-Sepharose. Thyroglobulin is a glycoprotein rich in  $\alpha$ -galactose carbohydrate. IgG and IgM subclasses were separated in a second step by gel filtration (this procedure fractionates on the basis of size).

10 A selection of free and conjugated sugars was tested for ability to inhibit the binding of the XNA IgM to an  $\alpha$ 1,3-galactosylated glycoprotein (referred to herein as "B-trisaccharide-HSA". This is a construct comprising a plurality of Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc moieties (i.e. B-

15 trisaccharides) linked to human serum albumin through three atom spacers, and is available from Dextra Laboratories Ltd, Reading, UK, under product number NGP2334) (Figure 1). The most striking result was that B-trisaccharide-HSA was a hundred-fold more inhibitory

20 towards IgM binding than unconjugated Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc (referred to in Figures 1 and 3 as "B-trisaccharide"). These figures also refer to "B-disaccharide". This is Gal $\alpha$ 1,3Gal. In some cases this is used with a spacer arm. The spacer arm is

25 OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>. Figure 1 refers to B-disaccharide-PAA. This is a conjugate of polyacrylamide and a plurality of Gal $\alpha$ 1,3Gal epitopes and polyacrylamide, as described in Rieben et al, *Xenotransplantation* 2 98-106 (1995). Each disaccharide epitope is linked to a CONH moiety on the

30 polyacrylamide through a spacer of the composition CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>O. It is obtainable from Syntesome GmbH and was used for comparison with B-trisaccharide-HSA. A 10% mol substitution form was used.

005101" 64226960



5 The ability of affinity-isolated human IgG and IgM to  
trigger lysis of porcine endothelial cells was determined  
by a two-stage <sup>51</sup>chromium release assay. Briefly normal  
porcine endothelial cells were grown to confluence in 96-  
well plates before being washed and labelled with  
<sup>51</sup>chromium. Excess label was removed and the cells were  
exposed to different concentrations of XNA. Unbound  
antibody was removed and 1/8 baby rabbit complement added  
for 1 hour at 37°C. Cell lysis was expressed as a  
10 percentage of the total counts associated with the  
endothelial cells, which could be detected in the medium  
after incubation of the cells with complement.

15 Both immunoglobulins bound to the endothelial cells  
resulting in cell lysis through their fixation and  
subsequent activation of the baby rabbit complement. In  
control experiments antibody or baby rabbit complement  
alone resulted in no specific cell lysis (Figure 2). The  
IgG fraction diluted out to 12.5µg/ml whereas the value  
20 for IgM was 0.47µg/ml. There was an approximate ten-fold  
difference in the values for 50% lysis, c.32µg/ml for IgG  
compared to 3.2µg/ml for IgM.

25 Antibody binding (and hence lysis) to the porcine  
endothelial cells was then inhibited with various sugars.  
Affinity-isolated IgM (10µg/ml) or IgG (80µg/ml) was  
incubated with endothelial cells as before in the  
presence of varying concentrations of four different  
sugar preparations. The cells were then washed and  
30 exposed to baby rabbit complement as previously  
described.

The most potent inhibitor of cell lysis and therefore  
antibody binding in the presence of either immunoglobulin

005707" 64225950

5 was the B-trisaccharide-HSA conjugate (Figure 3). As an  
 inhibitor of XNA IgM binding this was over ten-fold more  
 effective when compared to the unconjugated B-  
 trisaccharide which in turn was superior to B-  
 disaccharide (i.e. Gal $\alpha$ 1,3Gal) in the presence of IgM.  
 10 The effect was less marked for IgG but still significant.  
 There was minimal difference between B-trisaccharide and  
 B-disaccharide for IgG binding, however they were more  
 effective against IgG as opposed to IgM in their  
 inhibitory action. Glucose, employed as a non-specific  
 sugar, had no inhibitory action on either IgG or IgM  
 binding even at a concentration of 10 mM.

15 The B-trisaccharide-HSA conjugate used was a very  
 efficient inhibitor of the destruction of porcine cells  
 elicited by the application of human XNA *in vitro*.

20 This compound could prolong the survival of pig-human  
 xenotransplants, and since fewer molecules are likely to  
 be required to give protection (compared to free  $\alpha$ -  
 galactosylated sugars), osmotic disturbances in the  
 recipient may be diminished.

25 Further data supporting the present invention is provided  
 in Tables 1 and 2 and in Figures 4 to 7. This data is  
 discussed at pages 16 to 21.

#### List of Abbreviations Used

30 BSA: bovine serum albumin  
 HSA: human serum albumin  
 Glc: glucose  
 GlcNAc: N-acetylglucosamine  
 GalNAc: N-acetylgalactosamine

006707" 64226960

Gal: galactose

XNA: xenogenic natural antibodies

IgG: immunoglobulin of the G class

IgM: immunoglobulin of the M class

5 B trisaccharide-HSA: A plurality of Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc  
epitopes conjugated to human serum albumin

DME: Dulbecco's Modified Eagle's Medium

006707" 6426560

TABLE 1

antibody source	anti-A	anti-B
anti-A mAb	1:800	<1:50
anti-B mAb	<1:50	1:1600
544 pre-Tx	<1:40	<1:40
w544 Tx + 17 days	<1:40	<1:40
w544 Tx + 24 days	<1:40	<1:40
w544 Tx + 27 days	<1:40	<1:40
w544 Tx + 34 days	<1:40	<1:40
w141 pre-Tx	<1:40	<1:40
w141 Tx + 21 days	<1:40	<1:40
w141 Tx + 29 days	<1:40	<1:40
w135 Pre-Tx	<1:40	<1:40
w135 Tx + 34 days	<1:40	<1:40
w135 Tx + 36 days	<1:40	<1:40
w135 Tx + 37 days	<1:40	<1:40
T381 pre-Tx	1:320	<1:40
T381 + 2 days	<1:40	<1:40
T381 + 5 days	1:320	<1:40
T381 + 8 days	1:320	<1:40
V337 pre-Tx	<1:40	<1:40
V337 + 1 day	1:320	<1:40
V337 + 4 days	<1:40	<1:40
V337 + 7 days	<1:40	<1:40

006707" 64225950

TABLE 2

sample	reduction in IgG binding (%)	reduction in IgM binding (%)
W544-pre	14.5	17.4
W544-13 days	0	14.9
W544-20 days	0	12.5
W141-4 days	0	20.2

006T0T" 64226950